

IMAGING PLATFORM FOR NANOPARTICLE DETECTION APPLIED TO SPR BIOMOLECULAR INTERACTION ANALYSIS

Related Applications

This application is based on a prior copending provisional application,
5 Serial No. 60/451,346, filed on February 27, 2003, the benefit of the filing date of
which is hereby claimed under 35 U.S.C. § 119(e), and is also a
continuation-in-part of a prior copending application, Serial No. 10/628,662, filed
on July 28, 2003, which itself is a continuation-in-part of a prior copending
application, Serial No. 09/976,257, filed on October 10, 2001, the benefits of the
10 filing dates of which are hereby claimed under 35 U.S.C. § 120.

Field of the Invention

The present invention generally relates to the field of imaging objects
entrained in a flow of fluid, and more specifically, relates to imaging objects such
as individual nanoparticles that are entrained in a flow of fluid to obtain surface
15 plasmon resonance (SPR) spectra corresponding to the objects.

Background of the Invention

SPR is the resonant excitation of oscillating free charges at the interface of
a metal and a dielectric. When SPR spectra are generated and collected, they can
be used to determine specificity, kinetics, affinity, and concentration with respect
20 to the interactions between two or more molecules, where one of the molecules is
attached to a solid sensing surface. Reaction kinetics correspond to both an
association and a dissociation rate at which an analyte interacts with the bound
detection molecule. Affinity refers to the strength with which an analyte binds to
the detecting molecule. Specificity refers to the propensity of a molecule to bind
25 to the detecting molecule to the exclusion of other molecules. SPR spectra have

been used in studies involving many types of molecules including proteins, peptides, nucleic acids, carbohydrates, lipids, and low molecular weight substances (e.g., hormones and pharmaceuticals).

One analytical technique, known as SPR based bio-sensing, has been developed to enable direct measurements of the association of ligands with receptors, without the use of indirect labels, such as fluorescent markers and radioactive molecular tags. This label free direct sensing technique reduces the time and workload required to perform assays, and minimizes the risk of producing misleading results caused by molecular changes induced by the use of indirect labels. Another important aspect of the bio-sensing technique is that SPR based bio-sensing enables bio-molecular interactions to be measured continuously and in real-time, thereby enabling the determination of association and dissociation kinetic data in contrast to traditional “end point” analytical methods.

The utility and acceptance of SPR based bio-sensing is evident from the over 2,500 peer-reviewed scientific papers that have been published, which cite the use of SPR technology. To date, there is an estimated installed base of 1,500 research grade SPR analytical instruments in basic and applied research laboratories at universities, national research centers, and major pharmaceutical and biotechnology companies around the world. The diversity of recently published articles relating to bio-molecular interaction analysis include such applications as drug discovery (lead identification and target validation), ligand fishing, comparative binding specificity, mutation studies, cell signaling, multi-molecular complexes, immune regulation, immunoassays, vaccine development, and chromatographic development. Such SPR based research tools are of great value to researchers involved in basic and applied life sciences who are studying the function of molecules in biological systems.

Over the past decade, interest in the unique optical properties of metallic and semiconductor nanoparticles has increased considerably with respect to the use of suspensions and films incorporating these nanoparticles for the purposes of exciting surface plasmons to enable the detection of SPR spectra. In addition, surface enhanced Raman spectroscopy for infrared absorbance spectral

information and surface enhanced fluorescence for enhanced fluorescence stimulation can also be detected. Nanoparticles are particles that are less than 100 nanometers in diameter. They display large absorbance bands in the visible wavelength spectrum yielding colorful colloidal suspensions. The physical origin
5 of the light absorbance is due to incident light energy coupling to a coherent oscillation of the conduction band electrons on the metallic nanoparticle. This coupling of incident light is unique to discrete nanoparticles and films formed of nanoparticles (referred to as metallic island films). Achieving SPR with ordinary bulk materials requires the use of a prism, grating, or optical fiber to increase the
10 horizontal component of the incident light wave vector (i.e., to achieve the required coupling).

Historically, gold nanoparticles have been used as a pigment in stained glass as early as 350 years ago. Chemist and physicist Michael Faraday first recognized that the color of this stained glass was a result of the metallic gold
15 being in a colloidal form, and Gustaf Mie explained this phenomenon theoretically in 1908, by solving Maxwell's equation for absorption and scattering of electromagnetic radiation by a spherical particle.

Recently, sensor devices have been developed in the known art to exploit the unique optical properties of these nanoparticles. SPR measurements have
20 been made using gold nanoparticle suspensions to detect biomolecular interactions in real time by monitoring the absorbance of colloidal suspensions. Similarly, SPR has been excited using polystyrene and silica beads with silver and gold island films and hollow gold nanoshells. However, to date, all such measurements have been performed primarily on bulk homogeneous suspensions
25 of nanoparticles, due to the challenge of individually addressing and detecting these small objects.

For example, FIGURES 1A and 1B schematically illustrate a simplified version of a prior art SPR detection device including a single channel SPR bulk optic prism based sensor 10, which includes a prism 12. The base of prism 12 is
30 covered with a layer 14 of gold about 550 Ångstroms thick. The gold film is pre-functionalized with a defined detecting molecule 16, such as an antigen. A

biological fluid sample containing a corresponding analyte 32 (such as an antibody) is brought into contact with gold layer 14 and detecting molecules 16 by introducing the sample fluid into a flow cell 22 (note gold layer 14 and detecting molecules 16 together represent a sensor surface 20, which is in fluid communication with flow cell 22). A range of angles of monochromatic light 24 are directed towards and reflected from sensor surface 20. SPR arises through the coupling of energy between the incident photons of light with free electron oscillations ("plasmons") occurring at a gold film/liquid chemical sample interface at sensor surface 20. This interaction can cause a reduction in an intensity of reflected light 28 for a given angle 30, resulting in an absorbance or "resonance" dip 31 in a measured reflectance spectrum 34 (see FIGURE 1C). This resonance can also be observed in the wavelength domain if white light is introduced at an optimal fixed angle of incidence. When an analyte 32 binds to immobilized detecting molecule 16 on sensor surface 20 (see analyte 32a and molecule 16a of FIGURE 1B), the local mass concentration of molecules changes, which causes a corresponding change in the local refractive index close to sensor surface 20. The resultant increase in the refractive index causes a shift in the resonance angle, from angle 30 as illustrated in FIGURE 1A, to an angle 33 in FIGURE 1B. Angle 33 results in a "resonance" dip 35 in a measured reflectance spectrum 37, which is readily distinguishable from spectrum 34 (no binding event, so the reflectance angle is unchanged). Sensor 10 enables data collection to be performed continuously and in real-time, and some systems enable the user to observe the binding events in real time on a personal computer.

This bio-sensing technique was first reported in 1983, and first commercialized in 1990. Since then many different optical geometries have been explored including: (i) the Otto configuration, which utilizes an air gap between the optical coupling prism and the SPR supporting metal; (ii) the Kretschmann configuration, which eliminates the need for an air gap in favor of the metal film directly deposited upon the prism base; (iii) the use of a diffraction grating to excite SPR; (iv) an optical fiber configuration, wherein metal is deposited cylindrically around the fiber core; (v) planar/channel waveguide configurations

with retro-reflective elements; (vi) microstructure systems that have an integrated light source, detector, and guiding optics, including a capillary configuration in which SPR is excited in the interior capillary walls; (vi) use of gold island films; and (vii) two- dimensional (2D) imaging techniques for SPR array-based sensing.

5 FIGURE 1D graphically illustrates a typical SPR response curve 38 based on the association and dissociation of two bio-molecules. Curve 38 can be separated into four well-defined segments, each relating to a specific portion of an association/dissociation cycle. The portion of the cycle corresponding to segment A is schematically illustrated in FIGURE 1E. Flow cell 22 is in fluid
10 communication with detecting molecules 16, which are bound to gold layer 14. While no prism is shown in conjunction with FIGURES 1E-1H, it should be understood that the flow cells of FIGURES 1E-1H are used in with a prism, as shown in sensor 10 of FIGURES 1A and 1B.

Referring once again to FIGURE 1E, since analyte molecules are currently
15 not present in flow cell 22, there is no change in the angle of incidence in section A of spectrum 38 (FIGURE 1D). Generally the flow cell is filled with a buffer solution during this time period. Portion A of spectrum 38 is thus referred to as a baseline response.

In FIGURE 1F (corresponding to portion B of spectrum 38), molecules of
20 analyte 32 are introduced into flow cell 22 (i.e., a sample fluid containing the analyte is introduced into the flow cell). Some of the molecules of analyte 32 bind to detecting molecules 16, and the angle of incidence changes over time. Response curve 38 of FIGURE 1D typically represents a time period of about 5 to 20 minutes in duration. This response level indicates the baseline response.
25 During this “association” period, the analyte binds to the surface, thereby increasing the refractive index, causing the SPR resonant angle to increase (note the rise in portion B of spectrum 38).

In FIGURE 1G (corresponding to portion C of response curve 38), no
additional analyte is introduced into the flow cell. Instead, the flow cell is flushed
30 with a buffer solution. This step results in analytes being released from detection molecules 16, as the bound analytes 32 attempt to reach an equilibrium with the

buffer solution. The decrease in the amount of bound analyte is reflected in a dip in spectrum 38.

In FIGURE 1G (corresponding to portion D of spectrum 38), the flow cell is flushed with an acidic solution, which ensures that any residual bound analytes are removed from the detection molecules, thereby regenerating the sensor surface. This “regeneration” step enables the sensor surface to be returned to its original baseline configuration, so that further analyses can be performed. As noted above, the data collected during portions A-D of spectrum 38 (often referred to as a Sensorgram) enable the user to determine kinetics, concentration, binding specificity, and affinity.

FIGURE 2 shows a different prior art technique that has been developed, which also involves exciting and detecting SPR on gold and silver nanoparticles. Chemical and biological sensing applications using nanoparticles have been performed primarily by measuring a transmitted light intensity through a high concentration of suspended particles. The resultant spectrum has an absorbance dip due to the excitation of SPR at a certain coupling wavelength, as shown in FIGURE 2. The exact position and shape of the SPR spectrum is a function of such factors as the metal used, the bulk solution and adsorbed film complex refractive indices, the adsorbed film thickness, the nanoparticle morphology (size and shape), and inter-particle coupling effects (e.g. the concentration and proximity of nanoparticles to one another). For small nanoparticles compared to the wavelength, λ , the extinction cross section for the nanoparticles, can be approximated as indicated in Eq. 1, as follows:

$$\sigma_{ext} \approx \frac{9V\epsilon_b^{3/2}}{c} \cdot \frac{\omega\epsilon_2(\omega)}{[\epsilon_1(\omega) + 2\epsilon_b]^2 + \epsilon_2(\omega)^2} \quad (1)$$

where V is the spherical nanoparticle volume, c is the speed of light, ω is the angular frequency of the incident light, ϵ_b is the permittivity of the surrounding bulk dielectric medium (assumed to be relatively independent of the frequency of

light), $\epsilon_1(\omega)$ and $\epsilon_2(\omega)$ denote the real and imaginary parts of the metal permittivity, or more specifically, ($\epsilon(\omega) = \epsilon_1(\omega) + i\epsilon_2(\omega)$).

For nanoparticle SPR measurements, the maximum absorbance wavelength, λ_{spr} (SPR coupling wavelength) dependence on refractive index is not as sensitive as the bulk thin film SPR measurements. Sensitivity of a 75 nanometer shift in the SPR coupling wavelength per refractive index unit (RIU) is reported, as compared to 3000 nanometer shift per RIU for bulk film SPR devices. Thus, gold nanoparticle SPR measurement based methods are 40 times less sensitive. However, additional geometries, including gold/silver alloy nanoparticles, ellipsoidal nanoparticles, triangular nanoparticles, and hollow nanoshells have been reported as having increased sensitivities up to six fold (400 nm wavelength shift per RIU).

Although bulk SPR devices exhibit increased sensitivity to refractive index over SPR nanoparticle devices, the nanoparticles have an advantage with respect to the sensitivity of adsorption of molecules to the gold surface. Specifically, the decay length of the electric field extending from the gold/chemical sample interface is approximately 20 times shorter for that of nanoparticle colloidal gold versus bulk thin gold film. Therefore, because nanoparticles have more energy confined closer to the gold surface, these particles are more surface sensitive and will yield a larger signal during receptor/ligand interactions.

However, the above mentioned prior art techniques are currently limited by throughput, mass transport diffusion, and depletion of small concentrations of analytes. Commercial SPR biosensors are currently limited to four-channel detection. This fact, and the relatively high degree of training necessary to operate these instruments and analyze the results, currently limit SPR analytical use in the laboratory. In contrast, other bio-molecular analytical methods, such as immunological assays, and spectroscopic techniques (absorption, fluorescence spectroscopy, and fluorescent polarization) have kept up with increased analytical demands by making available instruments having, for example, 96, 384, and 1,536 micro-wells. It should be noted that there are several publications directed

towards multi-spot or 2D array SPR sensors. However, most if not all of these approaches are directed toward optical configurations that can only detect a single angle or single wavelength intensity. Therefore, changes in the association or dissociation of bio-molecules are detected as an intensity change, which has
5 limited sensitivity and limited dynamic range compared to full spectral SPR data, where the entire angular or wavelength spectrum is measured, enabling a high precision measurement of the coupling angle or wavelength.

Current commercial SPR instrumentation uses a fixed sensor having a gold layer capable of supporting SPR, such as the traditional SPR bulk optic prism
10 based sensor shown in FIGURES 1A and 1B. The bio-molecular receptor molecules attached to the gold layer are analyte specific. The analyte is brought to this sensor surface via fluidics, and the analyte associates with the bound receptor molecules on the sensor surface. Current planar embodiments are severely mass transport limited by diffusion to time scales on the order of 16 to
15 160 minutes for analytes at bulk concentrations less than 10^{-7} M.

Finally, current commercial SPR instrumentation uses sensors that have relatively large areas (e.g., four, twelve, and a hundred square millimeters). Because the SPR signal is proportional to the density of binding, having a large sensor area limits the analyte sensitivity, since low concentration analyte binding
20 serves to deplete the analyte concentration near the surface.

It would therefore be desirable to provide apparatus and a method that address and detect individual nanoparticles and particles, enabling high throughput and full spectrum SPR measurement, measuring the association of molecules free in solution via SPR emitted from nanoparticles and micro particles
25 suspended in solution, employing a significantly reduced sensor area with improved analyte sensitivity, providing a label-free direct sensing approach that reduces time and workload needed to carryout assays, and measuring biomolecular interactions continuously and in real-time. The prior art does not teach or suggest a complete solution to the problems discussed above.

Summary of the Invention

A first aspect of the invention is directed to an SPR biomolecular interaction method that uses flow imaging systems, which can combine the speed, sample handling, and cell sorting capabilities of flow cytometry with the imagery, sensitivity, and resolution of multiple forms of microscopy and full visible/near infrared spectral analysis of detector technology to collect and analyze SPR spectra from objects entrained in a flow of fluid that emit an SPR spectra. This method includes the steps of placing gold or silver nanoparticles or beads that have a detector molecule attached to them in a container, adding a solution of analyte or ligand molecules to the container, and introducing a portion of the sample into a flow imaging system. The flow imaging system provides up to a thousand-fold increase in signal collection over conventional SPR instrument designs and allows for maintaining particles in suspension to ensure optimal free solution conditions for association and dissociation of bio-molecular species. This approach is thus not severely mass transport limited, like planar embodiments. A peak absorbance wavelength can then be measured using detector technology. Since full spectral SPR data can be collected with this detector technology, the entire angular or wavelength spectrum is measured, providing a very precise measurement of the coupling angle or wavelength. In addition, this approach has the ability to measure libraries of different bead receptors. Also, this method includes repeating these steps on the portion of the sample that remains in the container. After centrifuging, removing the supernatant, and adding a buffer solution, this buffered portion of the remaining sample can be introduced into the flow imaging system, and disassociation rates can be studied. If desired, an optional step can be employed, wherein a low pH wash is used to remove the bound ligands from the receptors attached to the nanoparticles, and repeated measurements can then be made.

Corrections can be made to a nanoparticle response curve that exhibits a non-linear response. Specifically, larger nanoparticles may be used to increase the curve's linearity, and calibration corrections can be made for the non-linear response.

The preferred flow imaging systems can be used to analyze white light spectral scatter analysis of gold nanoparticles and nanoparticle-coated microbeads using prism dispersion. A prism or grating is employed to disperse laterally high resolution white light spectral scatter spectra of the nano or micro particles being
5 analyzed.

Yet another step of this method involves collecting simultaneous imaging of absorbed, scattered, and fluorescent light from microbeads.

Furthermore, if the prism is removed from the preferred flow imaging system and a focusing spherical lens is replaced with a cylindrical lens, the high
10 resolution scattered angular spatial intensity of the nano or micro particle can be measured under monochromatic side illumination.

Another aspect of this invention provides for multi-spectral darkfield scattering to analyze particles. For particles with sizes equal or smaller than the pixel size in the image plane, the size of such particle can be determined by
15 measuring the relative light scattering intensity across multiple wavelengths. The ratio of the scattered light intensities at given wavelengths is a function of the size of the particle, based upon Raleigh scattering.

Furthermore, a holographic notch filter can be used with the preferred imaging system to filter out the excitation laser light signal to detect surface
20 enhanced Raman spectroscopy.

Brief Description of the Drawing Figures

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same becomes better understood by reference to the following detailed description, when taken in conjunction with the
25 accompanying drawings, wherein:

FIGURE 1A schematically illustrates a prior art SPR sensor including a thin layer of gold and a prism;

FIGURE 1B schematically illustrates analytes being attracted to specific detection molecules attached to the gold film in the prior art SPR sensor of
30 FIGURE 1A;

FIGURE 1C is a graph illustrating the relationship between an intensity of light reflected from the gold layer through the prism and an angle of incidence with which such light is reflected using the prior art SPR sensor of FIGURE 1A ;

FIGURE 1D is a graph illustrating a characteristic relationship between a resonant angle and time based on a preferred analytical technique using the prior art SPR sensor of FIGURE 1A;

FIGURE 1E schematically illustrates a first step in a preferred prior art technique for using the prior art SPR sensor of FIGURE 1A;

FIGURE 1F schematically illustrates a second step in a preferred prior art technique for using the prior art SPR sensor of FIGURE 1A;

FIGURE 1G schematically illustrates a third step in a preferred prior art technique for using the prior art SPR sensor of FIGURE 1A;

FIGURE 1H schematically illustrates a fourth step in a preferred prior art technique for using the prior art SPR sensor of FIGURE 1A;

FIGURE 2 schematically illustrates a prior art technique for obtaining a transmission measurement from a bulk solution of gold nanoparticles;

FIGURE 3 is a schematic illustration of a preferred flow imaging system used in accord with the present invention;

FIGURE 4 is a schematic illustration of a readout provided by a time delay integrated (TDI) detector employed in a preferred imaging system used in accord with the present invention;

FIGURES 5A-5G schematically illustrate a method for using the flow imaging system of FIGURE 3 and the detector technology of FIGURE 4 for biomolecular interaction analysis, in connection with nanoparticle or micro particle SPR substrates;

FIGURE 6 is a flow chart showing the steps of the method illustrated in FIGURE 5A-5G, including an additional step for facilitating repeated measurements;

FIGURE 7 is a schematic illustration of a theoretical SPR Coupling Wavelength shift for a bulk gold film and a gold nanoparticles as a function of adsorbed film thickness;

FIGURE 8A is a schematic illustration of yet another preferred imaging system, which incorporates a prism for full spectrum analysis;

FIGURE 8B schematically illustrates data segments collected using the imaging system of FIGURE 8A;

5 FIGURE 9 schematically illustrates additional data segments collected using the imaging system of FIGURE 8A;

FIGURE 10A is a schematic side elevational view of an imaging system including a system of lenses in place of the prism employed in the imaging system of FIGURE 9;

10 FIGURE 10B is a schematic plan view of an imaging system including a system of lenses in place of the prism employed in the imaging system of FIGURE 9;

FIGURE 11 is a multi-spectral darkfield scatter analysis of ten continuous segments, 350 nm diameter beads; and

15 FIGURE 12 is a schematic illustration of yet another preferred imaging system including a prism, which is particularly well adapted to be employed for surface enhanced Raman spectroscopy detection.

Description of the Preferred Embodiment

20 The present invention encompasses a method of using flow imaging systems that can combine the speed, sample handling, and cell sorting capabilities of flow cytometry with the imagery, sensitivity, and resolution of multiple forms of microscopy and full visible/near infrared spectral analysis to collect and analyze SPR spectra from objects entrained in a flow of fluid that emit an SPR spectra. Conventional methods of collecting and analyzing SPR spectra either
25 employ a fixed sensor that emits SPR spectra as a solution of particles interacts with a fixed sensor, or emits a combined spectra from a bulk solution of particles that individually emit SPR spectra. The fixed sensor embodiment is widely used, but has a limited throughput. The spectra collected from the bulk solution does not enable spectra from individual particles to be discerned. In contrast, the
30 present invention enables SPR spectra from individual particles to be collected

with a much greater throughput than achievable using the fixed sensor prior art techniques (discussed above in connection with FIGURES 1A-1H).

Before discussing the steps employed in a preferred method in accord with the present invention, it will be beneficial to review a flow imaging system that is preferably used to execute the method. FIGURE 3 illustrates the key components of an optical system employed to project light from objects in flow onto a detector (FIGURE 4) that employs an exemplary readout for any particle. Objects are hydrodynamically focused into a single-file line in the fluidic system, forming a tall but narrow field of view. This approach enables the lateral dimension of the detector to be used for signal decomposition.

Referring now to FIGURE 3, objects 99 are hydrodynamically focused in a flow of fluid directed into a flow cuvette 116 and illuminated from one or more sides using light sources 98 and 100. Light is collected from the objects with a high numerical aperture objective 102, and the collected light is directed along a light path including lenses 103 and slit 105. A fraction of the collected light is transmitted to an auto focus subsystem 104 and a velocity detection system 106. It should be noted that velocity detection system 106 is important to ensure the data acquired by the detection system, which are integrated over to time, increase the signal and are properly synchronized to the flow of fluid through the imaging system. In the context of the present invention, the objects are nanoparticles and micro particles including gold/and or silver film to enable SPR spectra to be collected. It should be understood that the imaging system can be used to image a wide variety of object types, including but not limited to biological cells and beads.

The majority of the light is passed to a spectral decomposition element 108. The decomposition element employs a fan-configuration of dichroic mirrors 110 to direct different spectral bands laterally onto different regions of a TDI detector 114. Thus, the imaging system is able to decompose the image of a single object 118 into multiple sub-images 120 across detector 114, each sub-image corresponding to a different spectral component. In this view, detector 114 has been enlarged and is shown separately to highlight its elements.

Spectral decomposition greatly facilitates the location, identification, and quantification of different fluorescence-labeled bio-molecules within a cell by isolating probe signals from each other, and from background auto-fluorescence. Spectral decomposition also enables simultaneous multimode imaging (brightfield, darkfield, etc.) using band-limited light in channels separate from those used for fluorescence. FIGURE 3 illustrates a typical flow-based embodiment of a flow imaging system. However, inset 101 in this Figure illustrates a plate-based embodiment of an imaging system that can be used in place of the flow-based embodiment.

Alternatively, the flow imaging system can employ a prism (as shown in FIGURE 8A) or a grating oriented to disperse light laterally with regard to the axis of flow prior to the final focusing optics, for spectral analysis of each object's intrinsic fluorescence. In yet another embodiment of a suitable flow imaging system, a cylindrical final focusing lens (see FIGURE 10A) can be employed to image a Fourier plane on the detector in the cross-flow axis, enabling analysis of the light scatter angle. These techniques for multi-spectral imaging, flow spectroscopy, and Fourier plane scatter angle analysis can be employed simultaneously by splitting the collected light into separate collection paths, with appropriate optics in each light path. For enhanced morphology or to analyze forward scatter light, a second imaging objective and collection train can be used to image the particles through an orthogonal facet of the flow cuvette 116, thereby viewing the objects in stereoscopic perspective with no loss of speed or sensitivity.

Turning now to FIGURE 4, detector 114 of the flow imaging system shown in FIGURE 3 uses a TDI that performs high throughput imaging with high sensitivity. As shown in an exemplary readout 138, the image on the TDI detector is read out one row of pixels at a time from the bottom of the detector. After each row is read out, the signals in the remaining detector pixels are shifted down by one row. The readout/shift process repeats continuously, causing latent image 142 to translate down the detector during readout (note the movement of latent image 142 through frames T1-T6). If the readout rate of the detector is

matched to the velocity of the object being imaged, the image does not blur as it moves down the TDI detector. In effect, the TDI detector electronically “pans” to track the motion of an object being imaged. The key to this technique is to accurately measure the velocity of the objects being imaged and to employ that measurement in feedback control of the TDI readout rate. Thus, accurate velocity detection for objects moving in flow enables the TDI imaging to be implemented properly.

One primary advantage of TDI detection over other approaches is the greatly increased image integration period it provides. A preferred flow imaging system used in connection with the present invention includes a TDI detector that has 512 rows of pixels, giving rise to a commensurate 500X increase in signal integration time. This increase enables the detection of even faint fluorescent probes within cell images and intrinsic auto fluorescence of cells acquired at a high-throughput. When applied to nanoparticles in suspension in a cuvette, real-time triggering and isolation of certain nanoparticle receptor/ligand combinations for post capture analysis can be performed. For example, selective retrieval of proteins from a complex biological sample in real time can be monitored. By isolating the nanoparticle receptor/ligand combination, mass spectroscopy can be used for identity confirmation of the affinity retrained analyte via its unique molecular mass.

Furthermore, the use of a TDI detector increases measured signal intensities up to a thousand fold, representing over a 30 fold improvement in signal-to-noise ratio compared to other approaches in the prior art. This increased signal intensity enables individual particles to be optically addressed, providing high resolution measurement of either scattered spectral intensity of white light or scattered angular analysis of monochromatic light. The ability to optically address individual particles, without requiring a prism to be disposed immediately adjacent to a thin metal film significantly distinguishes the use of the preferred imaging system of FIGURE 3 from the prior art SPR sensor of FIGURE 1. The sensor component in FIGURE 1 (the gold layer and the detector molecule) are fixed, whereas the method of the present invention employs a flow imaging

system similar to that illustrated in FIGURE 3, so that the “sensor” providing the SPR spectra is the gold or silver film deposited individually on particles imaged by the system. As noted above, this technique dramatically reduces the size of the detector surface, enabling more accurate data collection to be achieved.

5 A flow imaging system used in the present invention can be configured for multi-spectral imaging and can operate with six spectral channels: DAPI fluorescence (400-460 nm), Darkfield (460 - 500 nm), FITC fluorescence (500-560 nm), PE fluorescence (560 - 595 nm), Brightfield (595 – 650 nm), and Deep Red (650 – 700 nm). The TDI detector can provide 10 bit digital resolution per
10 pixel. The numeric aperture of the imaging system used with this invention is typically 0.75, with a pixel size of approximately 0.5 microns. However, those skilled in the art will recognize that this flow imaging system is neither limited to six spectral channels nor limited to either the stated aperture size or pixel size and resolution.

15 The SPR biomolecular interaction method of the present invention, which uses an imaging system (or a substantially similar imaging system), as described above, to image nanoparticles and larger particles having a metal film will now be described in detail. The method of the present invention benefits from the ability of this preferred flow imaging system to optically address and measure individual
20 SPR spectra of nanoparticles and larger sized particles in flow, resulting in up to a thousand-fold increase in signal collection over conventional SPR instrument designs. The steps involved in this method are schematically illustrated in FIGURES 5A-5H, and these same steps are shown as a flow chart in FIGURE 6. Note that the flow chart of FIGURE 6 includes an optional step that enables a user
25 to repeat measurements, if so desired.

Referring now to FIGURE 5A, in one embodiment of the method of the present invention, a solution 162 of gold coated nanoparticles 168 is introduced into a container 164. The gold coated nanoparticles have been functionalized with a “detecting” or receptor molecule attached to the surface of the gold
30 nanoparticles. Instead of nanoparticles, gold island microbead films can be employed. Gold is preferred, but silver coated microbeads and nanoparticles are

also known to enable SPR spectra to be generated and can instead be used with this invention. Combinations of gold and silver films can also be employed. Initially, container 164 has a minimal amount of a buffer solution, so as to minimize the dilution of solution 162. Preferably, any buffer solution added to
5 container 164 should be chemically consistent with solution 162 (i.e., if solution 162 is a saline solution, any additional buffer should be a saline solution) to minimize bulk refractive index effects. It should be noted that container 164, which includes solution 162 (with functionalized gold nanoparticles, or larger particles including receptors molecules), generally corresponds to flow cell 22 in
10 FIGURE 1E. Flow cell 22 in FIGURE 1E includes a functionalized gold sensor, but no analytes. Significantly, container 164 includes a plurality of individual particles, each particle acting as an individual sensor, while flow cell 22 of FIGURE 1E includes only a single sensor.

Those skilled in the art will recognize that container 164 may be of any
15 type and size capable of holding the solution, including but not limited to a beaker or test tube. Furthermore, it should be understood that FIGURES 5A-5H are not drawn to scale. For example, the amount of solution shown in the vial and its size may vary.

In a second step, schematically illustrated by FIGURE 5B, an analyte
20 solution 170 (i.e., a solution including a concentration of analyte or ligand molecules that will associate with the detection molecules bound to the individual particles in solution 162) contained in a second container 173 is added to solution 162 in container 164. While solution 170 is shown as being added as drops dispensed by a dropper, it should be understood that the present invention in
25 not limited to that specific implementation for transferring solution 170 to container 164. For example, solution 170 may be transferred into container 164 simply by pouring it, or by a more controllable technique, such as by utilizing a micro pipette to transfer the solution. Solutions 162 and 170 combine in
30 container 164 to form a sample solution 180, which includes gold covered nanoparticles, analyte molecules, and detector molecules bound to the nanoparticles.

In a third step schematically illustrated by FIGURE 5C, about one-half of sample solution 180 is removed from container 164 via a pipette 181 or other suitable transferring technique. The removed solution 180 is introduced into a flow imaging system, such as the imaging system discussed in detail above with respect to FIGURE 3. Preferably, the removed portion of solution 180 is introduced into a rotating syringe suspension pump (not separately shown). Such a pump serves to keep the particles in suspension via rotation, as well as enabling a precisely metering amount of sample to be introduced into the flow imaging system (i.e., into cuvette 116 as shown in FIGURE 3). As indicated above, a preferred imaging system requires small volume injection and very precise injection rates in order to maintain synchronization between the particulate flow rate and the TDI detector read out rate. Thus, a preferred syringe pump not only rotates the sample to maintain the particles in suspension, but also provides constant volume pumping with low pulsatility. Maintaining particles in suspension enables optimal *free solution* conditions for association and dissociation of bio-molecular species (i.e., the analyte molecules in solution 170) to the receptors/detector molecules on the gold nanoparticles in solution 162. Achieving such free solution conditions is a major advantage over prior art planar embodiments (i.e., as shown FIGURES 1A-1H and discussed above), which are severely mass transport limited by diffusion, to time scales on the order of 16 to 160 minutes for analytes at bulk concentrations less than 10^{-7} M.

In addition, the flow imaging system preferably employed uses hydrodynamic focusing (i.e., uses a sheath fluid) to confine a sample fluid (solution 180) to the central portion of a cuvette 116, as indicated in FIGURE 3. The sheath flow improves the precision with which the sample solution can be positioned in an observation region, enabling particles entrained in the flow of sample fluid to be more precisely imaged.

As a result of employing the flow imaging system described above to practice the method of the present invention, the absorption and/or reflected spectra of *individual* nanoparticles is readily measured using the TDI detector technology described in connection with FIGURE 4. A peak absorbance

wavelength is determined using any one of the many known “minimum hunt” algorithms to maximize precision, such as center of mass, parabolic curve fitting, and auto correlation. FIGURE 5D schematically illustrates an output of this data processing, in which SPR coupling wavelength 186 is plotted as a function of time 188, during the association time period (typically between 10 and 20 minutes). Because the method of the present invention uses the flow imaging system’s capability for the collection of full spectral SPR data, the entire angular or wavelength spectrum is measured, which provides a very precise measurement of the coupling angle or wavelength. This benefit is a clear advantage over the prior art, where only a single angle or single wavelength intensity can be measured. Additionally, throughput rates for imaging of macroscopic objects using this preferred imaging system are approximately 100 objects per second. When such a flow imaging system is operated in either spectral or angular dispersion mode for nanoparticles (scattered light or fluoresced light), these rates can be increased to achieve imaging of over a thousand particles per second. Also, if only one type of receptor (i.e., detection molecule, such as molecules 16 in FIGURE 1A) is bound to the population of nanoparticles, then the result will be a single association kinetic binding curve. However, if many different nanoparticles (or larger sized particles supporting SPR measurements) have different types of receptor molecules, this method enables the determination of multiple kinetic association rates.

The ability to measure a library of receptor beads requires a system to identify the bead. This identification can be done in one of the following two ways. First, a library set of nanoparticles having different SPR absorption spectra can be created. This step can be carried out by using alloy nanoparticles composed of silver and gold. By adjusting the mole fractions of the alloy, up to a 150 nm separation can be achieved. Given that the kinetic association and dissociation rates are continuous, this approach enables the encoding of nanoparticles that have relatively close absorbance spectra separation (e.g., about 5 nm), so that a library of 30 beads can readily be created. Secondly, by providing gold or silver island film deposition on micron beads, bead on bead

labeling can be used to encode a bead library numbering in the millions, using multiple fluorescent channel imagery. In addition, the SPR spectrum can be measured in the angular domain by using spatial light scattering. Note, spectral data and darkfield image 190 shown in FIGURE 5D is exemplary of images collected from fluorescent data, and while image 190 does not illustrate SPR reflection spectra, similar images based on SPR data can be obtained using this preferred flow imaging system.

It should also be understood that by using either nanoparticles or larger microbeads as SPR sensor surfaces, the sensor area can be significantly reduced, which as noted above, is advantageous over prior art SPR sensors having larger surface areas, since a large sensor area limits the analyte sensitivity, because the SPR signal is proportional to the density of binding. Specifically, if 2 micron polystyrene beads are used with an SPR supporting gold island film, a total of 180,000 beads would allow a bead library of 100 different receptor beads, and a sub-population of 1,800 beads per receptor. This preferred flow imaging system enables one bead to be read per second over a 30 minute association/dissociation observation period. These 180,000 beads would have a cumulative sensor surface area of 0.57 square millimeters. Furthermore, if instead of island coated microbeads, 100 nanometer nano-spheres were used, the accumulative surface area would be 1.4×10^{-3} square millimeters.

Referring now to FIGURE 5E, the portion of solution 180 that remains in container 164 (the portion that was not introduced into the flow imaging system) is used to measure the dissociation rate kinetics. During the association time period (10-20 minutes -- the time period corresponding to portion B of FIGURE 1D), solution 180 remaining in container 164 also undergoes the same association kinetics as occurred in the portion of solution 180 that was introduced into the flow imaging system. Therefore, this bead population may be used to study the dissociation rate kinetics. To study disassociation rates, solution 180 remaining in the container is concentrated into a small portion, by centrifuging. The supernatant (i.e., the portion of the solution containing little or no particles) is removed to achieve a concentrated solution 180a of gold nanoparticles. A buffer

solution 194 is added to the concentrated solution in container 164, to achieve a solution 195 that is a mixture of buffer solution 194 and concentrated solution 180a.

5 Immediately after buffer solution 194 is added to container 164, solution 195 is removed from container 164 and introduced into the flow imaging system discussed in detail above, as schematically illustrated in FIGURE 5F. As noted above, solution 195 is preferably introduced into the flow imaging system using a rotating syringe pump.

10 Again, the flow imaging system generates absorption and/or reflected spectra data for each individual nanoparticles 168 in solution 195, generating spectral data as shown in FIGURE 5G. The peak absorbance wavelength is determined and plotted as a function of time during the dissociation time period (typically between 5 and 15 minutes). A further optional step (not shown in FIGURE 5) is necessary if the user wants to repeat the measurement. The user, 15 may utilize a low pH wash step in order to remove the bound ligands (i.e., the analytes) from the receptors attached to the gold nanoparticles, so that the nanoparticles can be used again.

FIGURE 6 is a flow chart of the method schematically illustrated in FIGURES 5A-5G. The method begins in a block 200 where a container is filled 20 with functionalized gold or silver particles. A functionalized particle is a particle in which a receptor or detector molecule (such as an antibody) is attached to a metal layer on the particle, thereby enabling SPR spectra to be generated. In a particularly preferred method, the particles are gold-plated nanoparticles. However, it should be understood that silver-coated nanoparticles, or 25 nanoparticles coated with mixtures of gold and silver, can alternatively be employed. Further, larger sized particles supporting metal island films (also capable of generating SPR spectra) can also be alternatively employed.

Next, an analyte to be studied is added to the functionalized gold nanoparticle solution in a block 202. Half of the sample is aspirated by the 30 preferred flow imaging system's rotational suspension pump for kinetic *association* analysis in a block 204. It should be understood that either more or

less than half of the solution can be used in this step; using about half of the solution ensures that some solution is left to study dissociation kinetics, as described above. Further, if desired, all of the solution can be used to study association kinetics, if no data are desired from disassociation kinetics measurements.

5 In a block 206, the preferred flow imaging system performs continuous spectral analysis of individual particles. It should be understood that modifications can be made to the preferred imaging system described in FIGURE 3, so long as the desired SPR spectral data are obtained by the resulting flow imaging system. Thus, it will be clear that the flow imaging system of
10 FIGURE 3 is merely exemplary of a system that is suitable and capable of obtaining the desired spectral data, but is not intended to be limiting of this invention.

In a block 208, the maximum absorbance wavelength versus time is
15 plotted. While such a plot is useful, it should be understood that the method does not require the data be thus processed immediately. Instead, the raw data can be collected for review and processing at a later time.

In a block 210, the sample solution remaining in the container to which the analyte was added is concentrated, and an additional buffer solution is added (as
20 discussed in relation to FIGURES 1D and 1F, the additional buffer is required to induce the disassociation because equilibrium drives the analytes attached to the receptor molecules bound to the nanoparticles and metal films into the buffer solution).

In a block 212, the remaining concentrated sample and the buffer solution
25 (see FIGURE 5E and 5F) is introduced into the preferred flow imaging system for kinetic disassociation analysis. The preferred flow imaging system performs continuous spectral analysis of individual particles in a block 214. Then, once again, the maximum absorbance wavelength versus time is optionally plotted in a block 216.

30 A decision block 218 determines if the functionalized gold nanoparticles will be reused for further analysis of additional analytes. If so, the gold

nanoparticles that have been analyzed by the flow imaging system are collected and rinsed with acid in a block 220 to remove any analyte molecules that remain bound to receptor molecules on the nanoparticles (see FIGURES 1D, portion D, and FIGURE 1H). If no additional analysis is required, the method is done.

5 FIGURE 7 graphically illustrates the SPR coupling wavelength shift 230 as a function of the adsorbed film thickness 232 for a bulk gold film 234 (solid line) and nanoparticle 236 (dashed line) configurations, respectively, showing that the sensitivity of the two SPR configurations are within a factor of two of one another. Nanoparticle response curve 236 exhibits a non-linear response 240,
10 since it represents the entire dynamic range of film thickness, and the non-linearity is due to the exponential decay of the electric field. In contrast, the bulk SPR sensor generates a substantially linear response. However, this nanoparticle response curve should not be considered as limiting the present invention.

 While a linear response is generally preferred, the non-linearity and
15 abbreviated dynamic range of nanoparticles response curve 236 should not be understood to mean that nanoparticle SPR spectra are not useful. Larger nanoparticles may be employed to increase the linearity of the curve, and calibration corrections can be made for the non-linear response. Significantly, most SPR sensorgrams do not utilize more than 40 nm in their response, but, as
20 long as a generally linear response in that range is achieved, such spectra are useful. .

 The calculations employed to generate the response curves of FIGURE 7 assume a bulk solution refractive index of 1.3336 (for water) and an adsorbed film refractive index of 1.45 (for proteins). The data calculated for bulk gold film
25 assume a prism material of BK-7, incident angle of 82 degrees, and a gold film thickness of 550 Ångstroms and were calculated with a software program using a matrix form of the Fresnel reflection coefficients. The data calculated for nanoparticle assume an enhanced nanoshell (exhibiting six-times enhanced signal shift) and employ an approximation of the SPR coupling wavelength dependence
30 described by Eq (2.0), as follows:

$$\Delta\lambda_{spr} \approx a \left[n_2^2 \left(1 - \frac{1 - (n_1/n_2)}{(1 + (d/r_o))^3} \right) \right]^2 - n_1^2 \quad (2.0)$$

Turning now to FIGURES 8A- 12, various additional embodiments of a flow imaging systems that can be used for the biomolecular interaction analysis of the present invention will be discussed. FIGURE 8A schematically illustrates how the preferred flow imaging system discussed in connection with FIGURE 3 can be modified to be used for white light spectral scatter analysis using prism dispersion. Such a modification enables detailed spectral characterization of both gold nanoparticles and nanoparticle coated microbeads. By replacing spectral decomposition filter stack element 108, shown in FIGURE 3, with a prism 290 or grating oriented to disperse laterally as shown in FIGURE 8A, high resolution white light spectral scatter spectra of the nano or micro particle under analysis can be obtained. Exemplary data obtained from the imaging system of FIGURE 8A are shown in FIGURE 9.

FIGURE 8B illustrates continuously segmented data 292 from eighteen 1 micron diameter fluorescently labelled particles. The range of wavelength detection is from 488 nm to 750 nm. Each particle is imaged in the darkfield at the 488 nm excitation wavelength, and additionally, the fluorescent spectral emission from each particle is determined. The bead set analyzed contained three separate populations with different fluorescent emission spectra, as illustrated by the top three data segments 296 of the data buffer. Data segment 298 (the 6th segment from the top) and data segment 300 (the 16th segment from the top) indicate the presence of two clumped beads. Data segment 302 (the 11th segment) in the fluorescent spectrum indicates the presence of three clumped beads. Thus, the preferred flow imaging system of FIGURE 3 can be readily modified as indicated in FIGURE 8A to achieve a tool for individually measuring the SPR spectra of nanoparticles.

FIGURE 9 schematically illustrates exemplary data collected from the preferred imaging system of FIGURE 3, modified as indicated in FIGURE 8A, where the resulting flow imaging system enables simultaneous imaging of

absorbed, scattered, and fluorescent light from objects in flow. While this configuration cannot image objects as small as nanoparticles (as opposed to collecting spectral data from such small particles), the configuration of the imaging system in FIGURE 8A does allow image data from microbeads to be
5 analyzed. As indicated above, metal island films exhibiting the SPR phenomena can be supported on micron-sized beads. Thus, the imaging system shown in FIGURE 8A can be used to decode bead-on-bead libraries of metal island micron-sized beads.

FIGURE 9 shows exemplary data 268 from a multispectral image data set
10 of fluorescent calibration beads generated by the flow imaging system of FIGURE 8A. Images of each bead 260 appear in a brightfield channel 262 and a darkfield channel 264, along with fluorescence images in a channel 266 in the channel corresponding to the dye present on each of the fluorescent calibration beads. This imagery was gathered at a magnification of 20X, corresponding to a
15 pixel size of approximately 0.65 microns at the object. The darkfield 264 imagery shows the lensing effect of each bead 260 due to its large index of refraction relative to the buffer solution. A scatter plot 270 in FIGURE 9 shows the discrimination power and sensitivity of the system. Though the TDI detector is linear in its response, high dynamic range is achieved, because each image covers
20 more than 50 pixels, each of which is digitized with 8-bit resolution. With typical cell samples and the 10-bit per pixel resolution of the TDI detector, over four decades of dynamic range is achievable.

FIGURE 10A is a side view, and FIGURE 10B is a plan view, showing how the imaging system of FIGURE 8A can be modified to enable scattered light
25 angular analysis of the biomolecular interaction analysis using nanoparticle SPR substrates described in connection with FIGURES 5A-5H and FIGURE 6. These modifications enable the detailed monochromatic angular scattered light intensity characteristic of both gold nanoparticles and nanoparticle coated microbeads. By removing dispersing prism 290 shown in FIGURE 8A and replacing the focusing
30 spherical lens 303 with a cylindrical lens 304 (FIGURES 10A and 10B), the high

resolution scattered angular spatial intensity of the nano or micro particle under monochromatic side illumination can be measured.

Specifically, monochromatic light 306 from a laser source 98 (FIGURE 8A) is used to illuminate a center 308 of cuvette 116. As a
5 nanoparticle 99 enters the field of view, the laser light is scattered along light paths 310, and the scattered light is collected and collimated by a sperical collection lens 312 to achieve collimated light 314. The collimated light is then focused by a cylindrical lens 304 upon TDI detector 114. The readout rate of the TDI detector is synchronized with the flow speed of nanoparticle 99, enabling up
10 to a thousand fold increase in signal intensity. The scattered angle spatial intensity distribution is measured along the pixelated row of TDI detector 114 (implemented as a charge coupled device (CCD) camera). For a numerical aperture of 0.75 (in air) for the collection lens, this arrangement enables the detection in a solid angle of 35 degrees, at which the SPR resonance spectra are to
15 be observed.

FIGURE 11 illustrates exemplary data collected from a early version of the flow imaging system of FIGURE 3, indicating how multispectral darkfield scattering can be used to analyze particles. For particles equal in size or smaller than the pixel size in the image plane, the size of such particles can be determined
20 by measuring the relative light scattering intensity across multiple wavelengths. The ratio of the scattered light intensities at given wavelengths is a function of the size of the particle based upon Raleigh scattering. FIGURE 11 illustrates continuously segmented data from ten unlabelled beads 350, each about 350 nm in diameter. The orthogonal scattered light images were collected using 488, 532,
25 670, and 780 nm laser excitation. Imagery in channel 352 was collected using a 455 nm laser excitation wavelength; imagery in channel 354 was collected using a 532 nm laser excitation wavelength; imagery in channel 356 was collected using a 760 nm laser excitation wavelength; and imagery in channel 358 was collected using a 780 nm laser excitation wavelength. Current implementations of the flow
30 imaging system of FIGURE 3 have been optimized for excitation wavelengths from 400-750 nm.

For nanoparticles that are small relative to the 0.25 micron pixel size of a flow imaging system, the image acts as a spatial noise filter, excluding the pixels outside the boundaries of the nanoparticles from integrated intensity calculations, thereby enhancing the signal-to-noise ratio. For example, assuming a pixel size of
5 0.25 μm , the measurement of absorbance intensity from a nanoparticle that spans three pixels in a fluorescence image will have approximately 100 times less background than a non-imaging system employing a 20 μm laser spot.

As discussed above, in addition to exciting and detecting SPR spectra from individual nanoparticles and nanoparticle film microbeads, nanoparticles have
10 also been shown to enhance various other optical processes, including Raman scattering and fluorescence through the resonance conditions due to the localized SPR. FIGURE 12 illustrates how the flow imaging system of FIGURE 8A can be modified to detect surface enhanced Raman spectroscopy. An optional holographic notch filter 309 is used to filter out the excitation laser light signal.

15 Although the present invention has been described in connection with the preferred form of practicing it and modifications thereto, those of ordinary skill in the art will understand that many other modifications can be made to the present invention within the scope of the claims that follow. Accordingly, it is not intended that the scope of the invention in any way be limited by the above description, but
20 instead be determined entirely by reference to the claims that follow.